



# Directional Spread of Alphaherpesviruses in the Nervous System

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Review

## Directional Spread of Alphaherpesviruses in the Nervous System

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**Abstract:** Alphaherpesviruses are pathogens that invade the nervous systems of their mammalian hosts. Directional spread of infection in the nervous system is a key component of the viral lifecycle and is critical for the onset of alphaherpesvirus-related diseases. Many alphaherpesvirus infections originate at peripheral sites, such as epithelial tissues, and then enter neurons of the peripheral nervous system (PNS), where lifelong latency is established. Following reactivation from latency and assembly of new viral particles, the infection typically spreads back out towards the periphery. These spread events result in the characteristic lesions (cold sores) commonly associated with herpes simplex virus (HSV) and herpes zoster (shingles) associated with varicella zoster virus (VZV). Occasionally, the infection spreads transsynaptically from the PNS into higher order neurons of the central nervous system (CNS). Spread of infection into the CNS, while rarer in natural hosts, often results in severe consequences, including death. In this review, we discuss the viral and cellular mechanisms that govern directional spread of infection in the nervous system. We focus on the molecular events that mediate long distance directional transport of viral particles in neurons during entry and egress.

**Keywords:** alphaherpesvirus; herpes simplex virus; pseudorabies virus; axonal transport; directional spread; cytoskeleton

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## 1. Introduction

### 1.1. Alphaherpesvirinae

Herpesviruses are characterized by a large double-stranded DNA genome, a complex enveloped virion and the ability to establish a latent phase as part of their lifecycle in their hosts [1]. The mammalian herpesviruses belong to the Herpesviridae family and can be classified into three subfamilies, including the alphaherpesvirinae, betaherpesvirinae and gammaherpesvirinae. Members of each subfamily are distinguished based on their genome content, the cell type where latency is established in the host and the length of their productive replication cycle [1]. Alphaherpesviruses have the broadest host range [1]. These viruses are pantropic and neuroinvasive [2,3]. Once in the peripheral or central nervous system, infection can spread within chains of synaptically connected neurons. This review addresses the molecular mechanisms underlying neuronal invasion and directional spread of alphaherpesvirus infections.

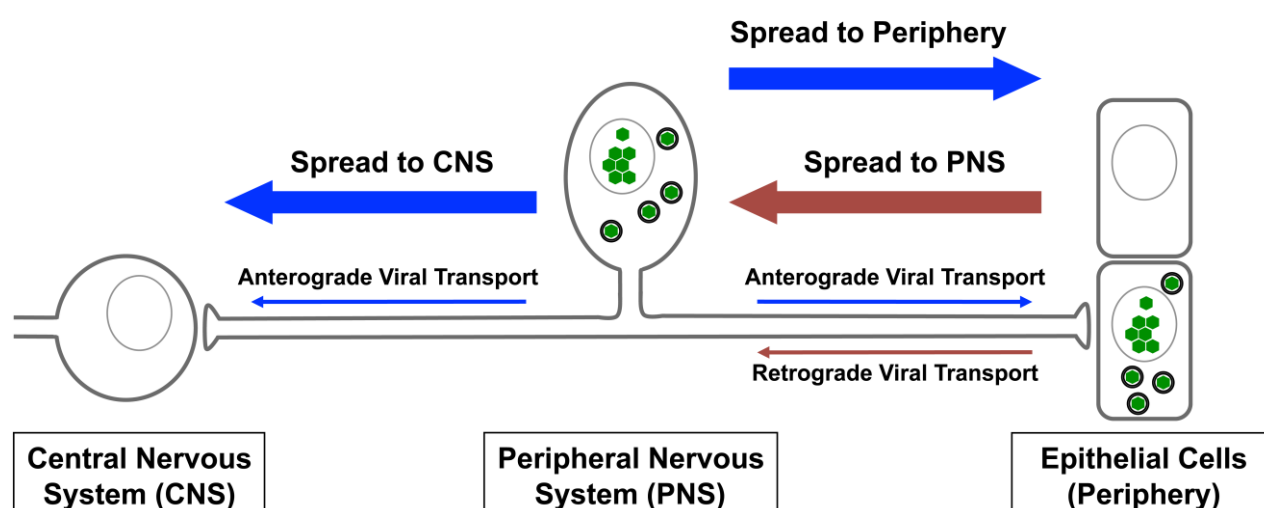
### 1.2. Model Alphaherpesviruses

The human alphaherpesviruses include herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) and varicella zoster virus (VZV). Furthermore, well-studied veterinary alphaherpesviruses include bovine herpesvirus type I (BHV-1), equine herpesvirus type I (EHV-1) and pseudorabies virus (PRV) [4]. Here, we focus primarily on HSV-1 and PRV, since both viruses have served as powerful models for understanding the molecular details of viral spread and pathogenesis, both *in vitro* and *in vivo*. In particular, several features make PRV an excellent model for studying alphaherpesvirus neuronal invasion and spread. First, PRV shares a similar genome structure to VZV, HSV-1 and HSV-2, and many of its gene products are functionally homologous to those encoded by these human pathogens [5,6]. Second, PRV can be used to infect a wide variety of experimental animal models and cell types. While its natural host is the adult swine, PRV infects a remarkably broad host range of vertebrates, with the exception of higher order primates, such as humans and chimpanzees [4]. Third, PRV readily grows to high titers and is easy to manipulate in the laboratory. Purified viral DNA or bacterial artificial chromosomes carrying the entire viral genome are infectious [7,8], and sophisticated molecular biological techniques to replace and manipulate PRV genes are well established. Fourth, working with PRV poses little to no risk for laboratory workers. Fifth, PRV serves as an excellent model for understanding the pathogenic consequences of alphaherpesvirus infections in animal models. Symptoms include severe pruritus and self-mutilation (known as the “mad itch”), as well as loss of motor coordination and ataxia. Infection of non-natural hosts with wild-type PRV is uniformly lethal [4]. Finally, because PRV spreads directionally and faithfully within chains of synaptically connected neurons, it has been used extensively as a powerful tool for studying the architecture of mammalian neuronal circuits [9].

### 1.3. Neuronal Architecture, Directional Spread of Infection and Alphaherpesvirus Pathogenesis

Spread of alphaherpesvirus infection occurs between synaptically connected neurons over long distances. Prior to entering the nervous system, alphaherpesviruses typically infect and replicate in somatic cells (such as epithelia) (Figure 1). Replication in these cell types is then followed by transmission of viral infection into neurons. To invade the nervous system of their hosts, viral particles enter nerve termini of the PNS and undergo retrograde transport towards the cell body, where they initiate a lifelong latent infection [10]. Following reactivation from latency, anterograde spread of infection is dependent upon axonal sorting and transport of viral particles back out towards the periphery (Figure 1) [11]. Occasionally, the infection can spread into the CNS, an event that is often associated with the fatal consequences of alphaherpesvirus infection, such as encephalitis [12]. Thus, targeted directional transport of viral particles in neurons, while poorly understood, is an integral part of the viral lifecycle.

**Figure 1.** Directional spread of alphaherpesvirus infection in the mammalian nervous system. In their hosts, alphaherpesvirus infections typically initiate at peripheral sites, such as mucosal epithelia. Next, viral particles enter at the termini of sensory neurons of the peripheral nervous system (PNS). These particles are transported long distances along axons in the retrograde direction towards cell bodies, where the genomes are deposited in the nucleus to establish lifelong latency. Following reactivation from latency, new viral particles are assembled and transported towards sites of egress. Typically, infections spread in the anterograde direction back out towards the periphery. This is essential for spread between hosts. Infection may also spread *trans*-neuronally, from the PNS to the central nervous system (CNS). Spread of alphaherpesvirus infection into the CNS is associated with lethal encephalitis.



As a consequence of their ability to spread from the PNS to the central nervous system (CNS) or to infect peripheral tissues of non-neuronal origin, alphaherpesviruses are the causative agents of a variety of neurological diseases and disorders [12]. In humans, HSV-1 and HSV-2 infections

commonly result in recurrent epidermal lesions (cold sores). Occasionally, infection may lead to more serious pathogenic consequences, such as meningoencephalitis, blindness and neonatal infections [13–15]. Additionally, VZV infection causes varicella (chickenpox) and herpes zoster (shingles) [16]. A variety of neurological complications are associated with VZV infections, including post-herpetic neuralgia, which is characterized by severe and constant pain in the affected dermatome [17]. Thus, the cellular and molecular underpinnings of alphaherpesvirus-inflicted neuronal damage, while poorly characterized, are central to understanding the etiology of symptoms and pathogenesis that occur as a result of infection.

Neurons are highly polarized cells with extended axonal and dendritic processes [18]. The directed trafficking of intracellular neuronal cargoes, such as ribonucleoprotein complexes (RNPs), mitochondria, synaptic vesicle precursors and other organelles, to specific subcellular domains is required to maintain both cell polarity and function [19,20]. During alphaherpesvirus infection, bidirectional transport of viral particles in axons and dendrites is essential for spread both within and between hosts [9,11]. Very little is currently known about viral particle dynamics in dendrites. The vast majority of studies have focused on sorting and transport of herpesvirus particles in axons during entry and egress [11,20,21].

*In vivo*, directional spread is likely dependent upon the architecture and connectivity of the neuron cell types that are typically infected. HSV-1, HSV-2 and VZV often establish latent infections in sensory neurons of the dorsal root and trigeminal ganglia [12]. These neurons are mostly pseudounipolar and, thus, have a bifurcated axon, such that one branch projects towards the periphery, while the other synapses with neurons of the CNS (Figure 1). Axonal sorting and anterograde transport of newly replicated particles is thought to proceed down both branches of these axons [11,22]. This presents an important conundrum. Following reactivation from latency, spread of infection towards the periphery is relatively common, whereas productive infection of the CNS is much rarer. Transsynaptic movement of viral particles likely occurs routinely from infected PNS neurons into higher order CNS neurons [23–25]. As evidence of this, HSV genomes are readily detectable in the CNS of infected rodents and humans [26–31]. Furthermore, Chen *et al.* demonstrated that latent HSV-1 and HSV-2 genomes could be reactivated from mouse brain stems, indicating that the CNS can be a latency site for HSV, with the potential to cause recurrent disease [23]. Similarly, latent PRV genomes have also been reported in brain tissue from adult swine, the virus's natural host [32–36]. Infection of mice with a virulent strain of PRV results in peripheral disease with minimal CNS invasion, while infection with an attenuated strain invades the CNS with minimal peripheral disease [37]. Thus, invasion of the PNS always follows infection of peripheral tissues, but spread to the CNS from the PNS depends on the extent of peripheral disease. Since both HSV-1 and PRV frequently gain access to the body of their natural hosts by infecting epithelial surfaces of the oronasal region, these viruses may also invade the CNS directly via the olfactory routes [38]. In brains of patients suffering from herpes simplex encephalitis, immunohistochemical analysis showed an enrichment of viral antigen mainly in the medial and inferior temporal lobes, hippocampus, amygdaloid nuclei, olfactory cortex, insula and cingulate gyrus [39]. This localization pattern would be consistent with entry of the virus via the olfactory pathway, with spread along the base of the brain to the temporal lobes. However, another possibility is that herpes simplex encephalitis may result from viral spread from the trigeminal ganglia to the temporal and frontal cortex, which would be consistent with the known site of

HSV-1 latency. Further work is needed to understand the molecular factors that determine the route of alphaherpesvirus neuroinvasion.

An important question is what are the viral or host factors that modulate or restrict the extent of viral neuroinvasion and replication in the CNS? An increasing amount of evidence suggests that the immune system plays a key role in this process by modulating reactivation from latency and viral gene expression. The innate response likely serves as the first line of defense. Toll-like receptors (TLRs) recognize pathogen-associated molecular patterns (PAMPs), such as those expressed by HSV-1. Activation of TLRs in neurons and glial cells leads to the production of cytokines that generate an antiviral response by recruiting macrophages or inducing proteins that degrade mRNA and inhibit translation. Similarly, TLR-dependent pathways are required for limiting HSV-1 replication in humans. Casrouge *et al.* previously reported that autosomal recessive mutations in UNC-93B, a transmembrane protein located within the ER, render children more susceptible to herpes simplex encephalitis by disrupting the interferon (IFN) response pathway [40]. UNC-93B functions by delivering TLR3, TLR7 and TLR9 from the ER to the endosome, the site at which they recognize PAMPs and initiate signaling cascades [41,42]. Recently, UNC-93B null patient-derived fibroblasts were differentiated into induced pluripotent stem cells (iPSCs) and then into CNS-resident cells, including neural stem cells (NSCs), neurons, astrocytes and oligodendrocytes. In this study, Lafaille *et al.* reported that UNC-93B-defective neurons and oligodendrocytes were more susceptible to HSV-1 infection compared to wild-type control cells [43]. Susceptibility was unchanged in NSCs and astrocytes. Intriguingly, similar results were obtained for cells derived from TLR3-defective neurons. These results suggest that signaling through the TLR3 pathway is necessary for efficiently modulating susceptibility to HSV-1 infection, specifically in neurons and astrocytes. This agrees with previous work indicating that TLR3-signaling is important for type I IFN responses and neuronal resistance to HSV-1 infection [44–46]. Collectively, these studies raise the possibility that cell types in the PNS and CNS are not equally susceptible and permissible to alphaherpesvirus infection. Furthermore, restriction of viral replication in the CNS by activation of TLRs may not be cell autonomous to CNS neurons. For example, Reinert *et al.* reported that TLR3 activation is responsible for restricting HSV-2 neuroinvasion by mediating the type I IFN response in astrocytes, thus limiting viral replication in CNS neurons [47]. The adaptive immune response also serves as an important line of defense against infections and functions to modulate viral spread in the nervous system. Viral specific T-cells are generated in response to infections within peripheral ganglia. These cells function to control viral replication and maintain latency. This topic has been recently reviewed in further detail [48].

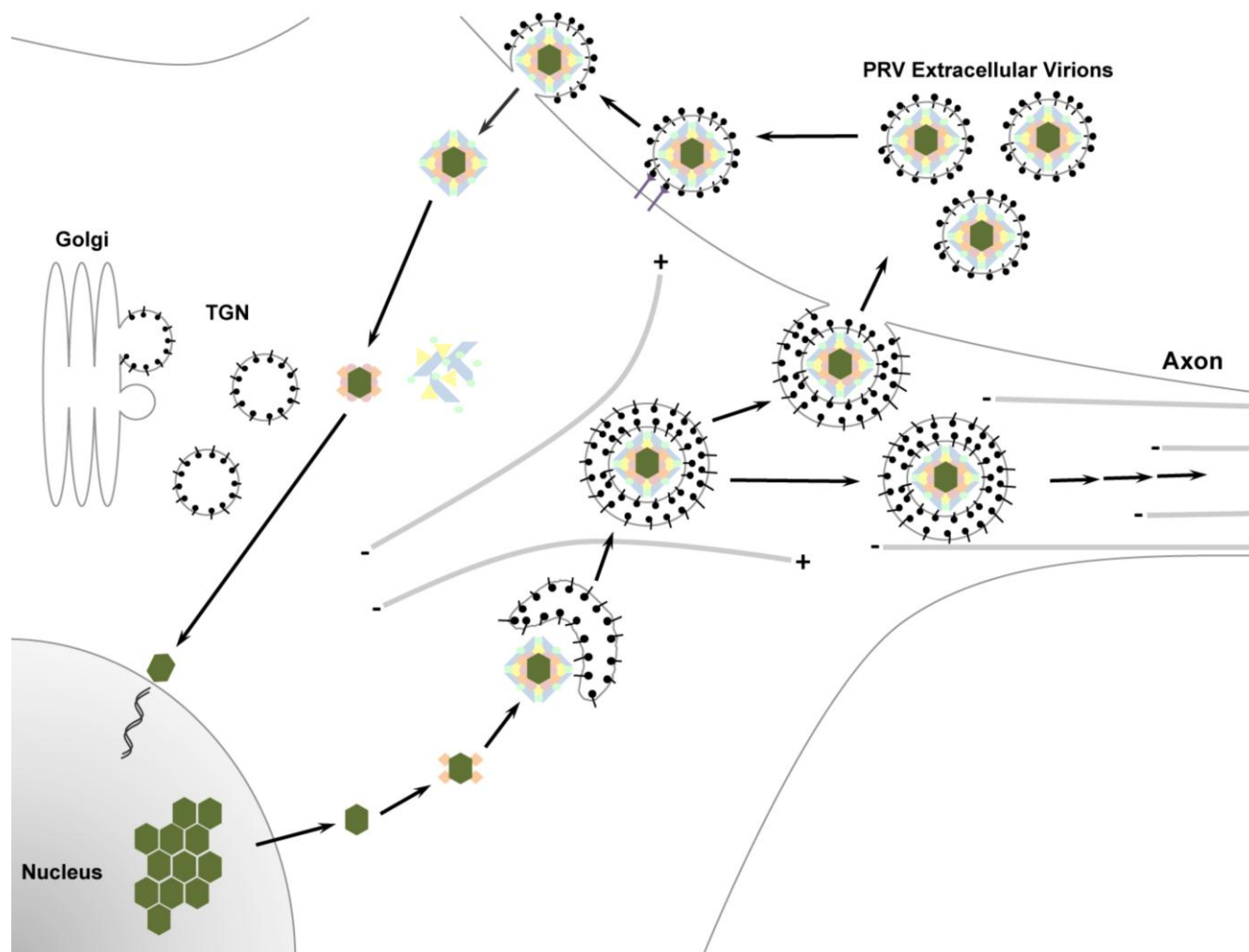
## 2. The Alphaherpesvirus Infectious Cycle

### 2.1. Virion Attachment and Entry

The directionality of spread is dependent upon the nature of the alphaherpesvirus infectious cycle (Figure 2). Infection is initiated by mature virions, which have a complex multilayered structure that is conserved among all herpesviruses [1]. At the core of the virion is a linear double-stranded DNA genome that is packaged within a proteinaceous capsid. The capsid is surrounded by a layer of viral

and cellular proteins known as the tegument, which is enclosed within a phospholipid bilayer studded with viral transmembrane proteins and is known as the virion envelope [4,49]. Infection is initiated by attachment of virions to heparan sulfate proteoglycans on the cell surface through the glycoproteins gC [50,51] and gB [52,53]. The next step of HSV-1 virion entry is dependent upon glycoprotein gD, which binds receptors on the surface of the plasma membrane, including Nectin-1, herpesvirus entry mediator (HVEM) and 3-O-sulfated heparan sulfate [54–57]. For PRV, gD is required for penetration of virions into cells, but not for cell-to-cell spread and neuroinvasion [58–60]. Although PRV gD binds Nectin-1 [61,62], neither Nectin-1 nor HVEM are required for PRV entry and spread in fibroblasts [60]. Thus, it is likely that there are other cellular receptors for PRV. VZV does not encode a gD homolog and likely depends on different viral proteins for interacting with entry receptors [63]. VZV gE may have gD-like functions during initiation of infection through binding the insulin-degrading enzyme (IDE), a proposed VZV receptor [64–66]. Following virion binding, glycoproteins gB, gH and gL, mediate fusion of the virion envelope with the plasma membrane [67–70]. Penetration of viral particles into the cell triggers the release of many of the tegument proteins into the cytoplasm [71,72]. However, a specific subset of “inner tegument” proteins (UL14, UL16, UL21, UL36, UL37, Us3 and ICP0) are thought to remain associated with capsids following entry [11,73]. These capsid tegument complexes are transported towards the nucleus [71,72]. Three of these proteins (UL36, UL37 and Us3) have been shown to co-transport with incoming PRV capsids by live cell imaging and electron microscopy [10,71,72,74,75]. Recent studies have focused on the role of the large-inner tegument protein, UL36, during viral entry. UL36 serves multiple functions during infection, including transport of capsids towards the nucleus [73,76], docking of capsids to the nuclear pore [74] and release of viral genomes into the nucleus [77]. Upon entering the nucleus, the linear viral genome becomes circularized. During productive replication, circular genomes serve as the template for DNA synthesis. The initial theta replication mechanism switches to a rolling-circle mechanism in order to produce long linear concatemeric genomes that serve as substrates for genome encapsidation [1,4]. Entry of the viral genome into the nucleus also results in the initiation of a highly temporally ordered viral gene transcription cascade that is characteristic of herpesvirus infection. Viral genes can be subdivided into three classes of successively expressed transcripts (immediate early, early and late genes) [1,4]. For PRV, gene transcription is initiated by expression of the immediate early gene IE180 (the homologue of HSV ICP4), a potent transcriptional activator that transinduces the expression of viral early genes [4,78]. Early genes primarily encode enzymes that are involved in nucleotide metabolism, while late genes encode structural components that are essential for genome packaging and assembly of new virions.

**Figure 2.** The pseudorabies virus (PRV) replication cycle. Following virion attachment and entry, viral capsids and tegument proteins are released into the cytoplasm. Many of the tegument proteins previously contained within the virion are released into the cytoplasm. Viral capsids and a specific subset of tegument proteins are trafficked towards the nucleus, where the viral genome is deposited and replicated. Capsids containing newly replicated viral genomes are released from the nucleus into the cytoplasm, where they further mature by acquiring viral and host tegument proteins. Final maturation occurs by envelopment of viral particles into vesicles derived from the trans-Golgi network (TGN) that contain viral and host membrane proteins. This process, known as secondary envelopment, results in a mature virion that is contained within a transport vesicle. Enveloped virions are trafficked towards sites of egress along the cell surface. In neurons, enveloped virions are sorted into axons and transported long distances along microtubules towards distal egress sites. At the target membrane, the transport vesicle and plasma membrane fuse, releasing a mature, enveloped PRV virion from the cell.





## 2.2. Genome Replication and Nuclear Egress

Newly replicated viral genomes are packaged into capsids in the nucleus. This process involves cleavage of the replicated concatemeric DNA into monomeric linear genomes and subsequent packaging of genomes into capsids [79,80]. After packaging, PRV nucleocapsids are released from the nucleus by crossing the nuclear envelope and then entering the cytoplasm. The molecular details of this process have been subject to debate over recent years [81]. The widely accepted model suggests that nuclear egress is initiated when nucleocapsids bud through the inner nuclear membrane into the perinuclear space through a process known as primary envelopment. This is followed by de-envelopment (fusion) at the outer nuclear membrane and release of the nucleocapsid into the cytoplasm [82,83]. Previously, this mode of nuclear egress was thought to be unique for herpesviruses. However, Speese *et al.* recently demonstrated that nuclear egress of large ribonucleoprotein particles (RNPs) occurs through a similar mechanism, suggesting that herpesvirus nuclear egress progresses by taking advantage of an existing cellular mechanism [84]. Nonetheless, efficient nuclear egress of herpesvirus nucleocapsids is dependent upon the viral proteins UL31, UL34 and Us3 [85–90]. Recent work has shown that additional viral tegument and membrane glycoproteins are associated with primary enveloped virions [91–94]; the putative roles of these proteins during nuclear egress remain poorly understood.

## 2.3. Virion Assembly, Secondary Envelopment and Cellular Egress

Following nuclear egress, virions undergo assembly and maturation in the cytoplasm, where they acquire tegument proteins and bud into vesicles that give rise to the final envelope [49]. Membrane acquisition of tegument-coated capsids occurs through a process known as secondary envelopment. This process produces enveloped virions that are contained within a transport vesicle. The cellular membranes used for secondary envelopment of PRV and HSV-1 have been shown to contain markers for the trans-Golgi network (TGN) [95–98]. However, it is also important to note that productive herpesvirus infection results in a dramatic rearrangement of membranous organelles, including the Golgi apparatus, TGN and endosomes, such that their subcellular localizations and boundaries become less well defined compared to uninfected cells [97,99]. Thus, while the exact cellular origin of membranes used for secondary envelopment remains unknown, these membranes contain a subset of viral and cellular proteins that facilitate virion assembly and egress.

For viral particles that are targeted for egress in axons, a lively debate has arisen regarding the assembly state of these particles prior to axonal sorting [11,100,101]. The “married model” holds that virion assembly and secondary envelopment occur in the cell body prior to axonal sorting and transport towards sites of egress at distal sites [83,102–118]. In contrast, under the “separate model” naked capsids (nucleocapsids that associate with a subset of tegument proteins, but not membranes) and secondary envelopment membranes undergo axonal sorting and transport separately. Final assembly of these components occurs at or near sites of egress [103,119–127]. Kratchmarov *et al.* recently presented a comprehensive review of the published evidence that supports each of these models [100]. An important conclusion is that overwhelming amounts of data support the married model during PRV

infection. Further work is necessary to assess whether axonal sorting and transport of HSV-1 follows the married or separate models or, potentially, a combination of both [103].

Virion egress occurs by fusion between the transport vesicle membrane and the plasma membrane to release virions into the extracellular environment. The molecular mechanism underlying these fusion events is unknown, but is likely dependent upon soluble N-ethylmaleimide attachment protein receptor (SNARE) proteins that mediate exocytosis of cellular vesicles [128–130]. Previous studies have demonstrated that HSV-1 and PRV particles are associated and co-transport with proteins that are involved in axonal secretory and exocytic pathways [105,120]. Furthermore, we recently found that the vesicle and target SNAREs (v-SNAREs and t-SNAREs), VAMP2, SNAP-25, VTI1B and Syntaxin-6 are incorporated with membrane microdomains that contain the PRV membrane protein Us9 [131]. As discussed below, since PRV Us9 is required for anterograde spread of infection, these cellular fusion proteins represent excellent candidates for mediating targeted virion egress in axons and dendrites.

### **3. Utilization of the Host Cytoskeleton for Directional Spread of Infection in Neurons**

#### *3.1. Viral Particle Movement Requires Active Transport*

Many viruses, including PRV and HSV, rely on interactions with the host cytoskeleton for effective spread and transmission. Diffusion is an inefficient means for long-distance directional movement of viral particles within the cytoplasm. An HSV capsid would take over 200 years to move 1 cm by diffusion alone [132]. This timescale is far too extended for efficient movement of viral particles in mammalian cells, let alone highly elongated cells, such as neurons that extend neurites millimeters or even meters away from their cell bodies. Thus, active transport via established cellular mechanisms is required for efficient viral infection and spread.

#### *3.2. The Actin and Microtubule Cytoskeletons*

Both the actin and microtubule cytoskeleton have well-established roles during herpesvirus infection [21,133,134]. Actin filaments (F-actin) play an essential role in short-distance cargo movement [135–138]. Filaments are composed of G-actin monomer subunits that form two protofilaments that wrap around each other [139]. This arrangement confers an intrinsic polarity consisting of “plus” (or “barbed”) and “minus” (or “pointed”) ends. Actin filaments range between 5 to 9 nm in diameter and often form three-dimensional networks within the cell, but are concentrated at the cell cortex [140]. Directed transport of cellular cargoes is mediated by the myosin superfamily of motor proteins [141]. Several members of this large superfamily of motors have been shown to be involved in targeting proteins to axons [142,143].

In contrast to the actin cytoskeleton, microtubules are required for facilitating long distance transport of cargo. Microtubules are stiff cylinders composed of alpha-tubulin and beta-tubulin heterodimeric subunits that assemble through head-to-tail association to form protofilaments [144]. Typically, 13 of these protofilaments join to form a hollow microtubule that has an outer diameter of 25 nm. Because of their composition, microtubule filaments possess an inherent structural polarity. Microtubules have a highly dynamic “plus” end that is often oriented towards the cell periphery, as

well as a relative stable “minus” end that is oriented towards the microtubule organizing center (MTOC) [145–147]. In neurons, the arrangement of microtubules is highly specialized. Axons contain microtubules that are oriented almost exclusively with plus-ends facing the axon terminus, while dendrites contain microtubules of mixed polarity [143,148]. This mode of organization is required for maintaining the identities of these distinct neuronal compartments. Two classes of molecular motor proteins mediate microtubule-dependent directional transport of cargoes. Dynein motors facilitate retrograde transport (towards the microtubule minus ends), while kinesin family motors facilitate anterograde transport (towards the microtubule plus ends) [149]. The kinesin family of motors is substantially more diverse; mammalian genomes can encode up to 45 different kinesin motors that are classified into 14 different subfamilies [143,150].

The actin and microtubule cytoskeletons often function cooperatively to maintain and facilitate proper cellular polarity and dynamics [151,152]. For example, the proximal axon is enriched in filamentous actin [153], as well as microtubule ends that are decorated with the microtubule end-binding (EB) proteins, EB1 and EB3 [154,155]. These structural components are anchored by the large scaffolding protein, ankyrin G, a key organizer of the axon initial segment [156]. Combined, these structural properties of the axon initial segment allow it to function as a selective filter for regulating transport of cellular components into the axonal compartment [153].

### *3.3. Retrograde Transport During Viral Entry*

To gain access to the cytoplasm, incoming viral particles must get past a layer of cortical actin [157]. While virion binding to cell surface receptors triggers rearrangement of the actin cytoskeleton [158,159], the importance of this event is currently unknown. Pretreatment of cells with the actin depolymerizing agent, cytochalasin D, does not affect the dynamics of viral entry [157]. In contrast, pretreatment of cells with the microtubule depolymerizing agents, nocodazole or colchicine, results in disruption of retrograde capsid transport towards the nucleus, demonstrating that the microtubule cytoskeleton is required for entry [22,157,160]. Live cell imaging of fluorescently labeled PRV and HSV-1 capsids in cultured neurons has shown that retrograde transport is a robust process that occurs at a rate of approximately 1  $\mu\text{m/s}$  [22,105,109,160–162]. This rate is consistent with fast axonal transport with the retrograde motor dynein [163]. Additionally, retrograde transport of capsids is salutatory and bidirectional, suggesting that kinesin motors may also be involved [105,162]. Both kinesin-1 and kinesin-2 have been shown to co-purify with partially tegumented capsids that were generated from extracellular virions and incubated in cytosolic extracts [73].

A small subset of viral capsid and tegument proteins have been implicated in recruiting motors that facilitate retrograde transport, including UL36, UL37 and VP26 (UL35) [73,74,76,164–167]. UL36 has emerged as the most likely candidate for direct motor recruitment to incoming virions. Expression of mutant UL36 proteins or injection of anti-UL36 antibodies results in a dramatic disruption of capsid delivery to the nucleus and, consequently, initiation of infection [74,76,166,168,169]. In contrast, deletion or mutation of UL37 or VP26 does not appear to have the same effect [165,170–172].

### 3.4. Anterograde Viral Transport and Axonal Sorting

In addition to its role during entry, UL36 is required for transport to the site of secondary envelopment and egress [173,174]. In the absence of UL36, PRV capsids are not transported along microtubules and do not undergo secondary envelopment. PRV deleted for UL37 is defective for secondary envelopment and displays reduced capsid transport (UL36 still binds capsids in UL37 null PRV) [76]. These observations suggest that UL36 and UL37 are key components of a motor protein recruitment complex that transports tegumented (or partially tegumented) capsids towards assembly sites. Presumably, these transport events depend primarily upon interactions with kinesin motors (as opposed to dynein). However, since the tegument compositions of newly produced nucleocapsids and incoming capsids appear to be similar (capsid proteins bound to UL36, UL37 and Us3), this leads to an apparent problem. How are capsids differentially targeted to the nucleus for entry or to sites of cytoplasmic envelopment for egress? Since UL36 has been shown to interact with motor proteins of opposite polarity (G.A. Smith, personal communication), it likely has the capacity to transport viral particles in both the anterograde and retrograde directions. Posttranslational modifications or binding of accessory viral tegument proteins are predicted to act as the “switch” between dynein and kinesin driven transport [175–180].

Following secondary envelopment in the cytoplasm, viral particles are further transported towards sites of egress along the cell periphery. This process is also sensitive to microtubule depolymerizing agents and, therefore, requires kinesin motors [125]. The specific viral and cellular proteins that mediate this process have not been previously identified.

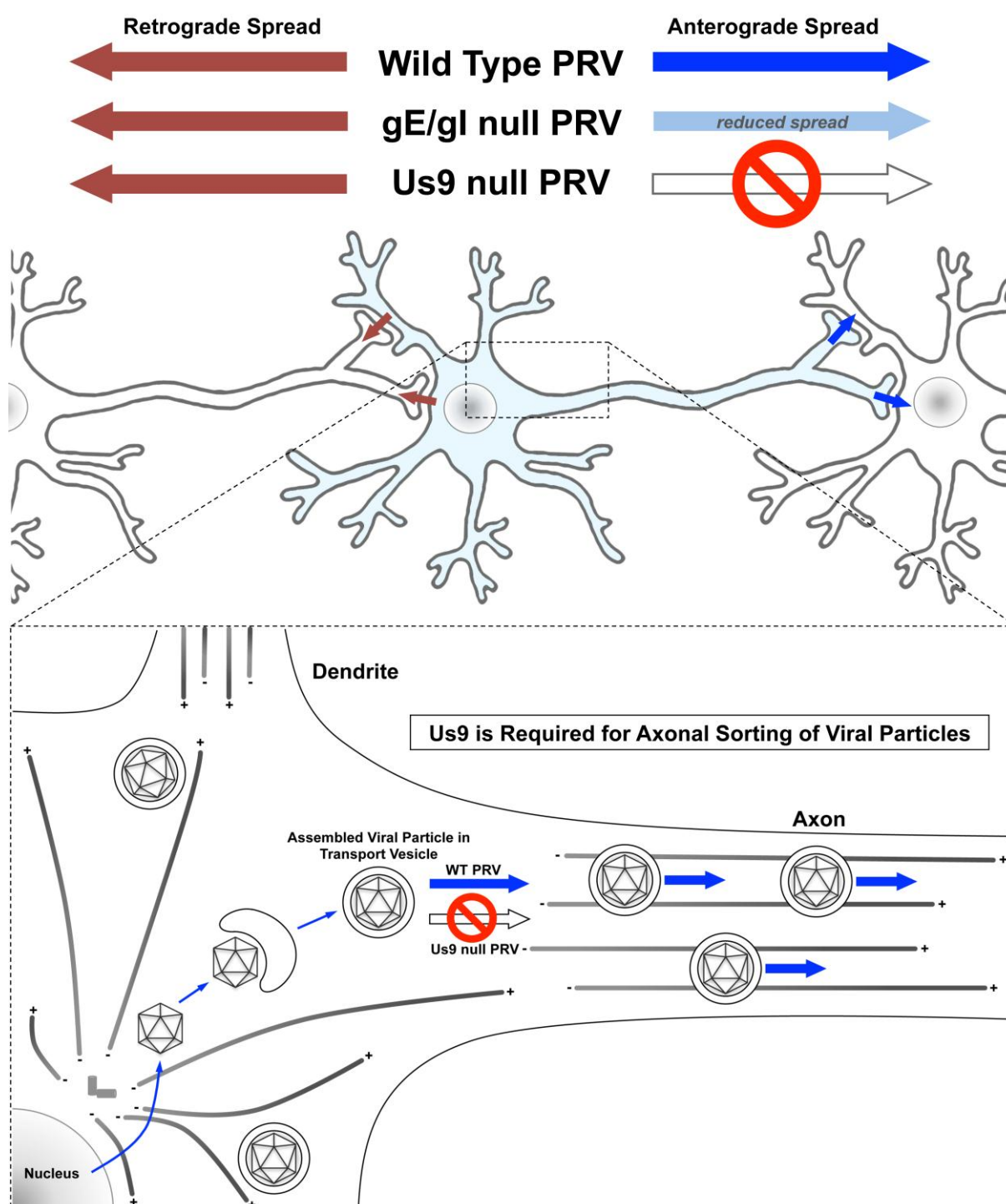
In non-neuronal cells, UL36 and Us11 have been proposed as candidate viral proteins for recruitment of kinesin motors during anterograde transport of enveloped viral particles [167,181–184]. Both proteins have been shown biochemically to interact with kinesin motor subunits. However, since UL36 and Us11 are tegument proteins, they are unlikely to partake in protein-protein interactions on the cytoplasmic surface of enveloped particles. One possibility is that a subset of tegument proteins, including UL36, may be membrane-associated and available on the outer surface of enveloped particles [185–188]. A second model holds that virions are partially enveloped, thus rendering the tegument exposed to cytosolic proteins [167]. Moreover, the potential involvement of Us11 in anterograde transport of enveloped virions poses additional caveats. First, Us11 is non-essential for viral replication. Second, Us11 is not conserved in all alphaherpesviruses, including PRV [182,183]. Thus, further work is needed to identify the viral and host proteins that facilitate anterograde transport of enveloped virions.

During neuronal infection, anterograde spread is dependent upon axonal sorting and long distance transport of viral particles. Anterograde-directed movement of viral particles is necessary for re-infection of innervated tissues following reactivation from latency and, consequently, successful spread between hosts. Three viral membrane proteins (gE, gI and Us9) are required for anterograde directed spread through neural circuits *in vivo* [37,189–196]. *In vitro*, the phenotype of individual gE, gI or Us9 null mutants are more varied (Figure 3). PRV gE and gI null mutants are able to undergo anterograde spread, but with a reduced capacity compared to wild-type [197]. Us9 null mutants display the most severe phenotype and are completely defective for anterograde transneuronal spread [108].

All three of these proteins are required for efficient targeting of virion structural components into axons [108,111,124]. gE/gI are both type I membrane proteins that form a heterodimer within the endoplasmic reticulum (ER) [195]. After leaving the ER, gE/gI are localized primarily to the Golgi apparatus, cytoplasmic vesicles and the plasma membrane [198,199]. By biotin labeling of cell surface proteins or using specific antibodies, our lab demonstrated that gE/gI undergo constitutive endocytosis from the plasma membrane and accumulate in large cytoplasmic vesicles. In porcine kidney epithelial cells (PK15), endocytosis of the gE/gI complex occurs early after infection, but stops completely after 6 hours post infection (HPI) [198]. Endocytosis of gE/gI is dependent upon the 123 amino acid cytoplasmic tail domain of gE (the full length protein contains 577 amino acids), especially a membrane proximal tyrosine-based internalization motif (at amino acids 478–481) [200,201]. Both HSV-1 and VZV gE also contain similar tyrosine-based motifs within their cytoplasmic domains that are important for endocytosis [200]. For PRV, this domain is not required for transneuronal spread of infection *in vivo* [199,201]. However, deletion of gE's cytoplasmic domain or the full length protein results in a small plaque phenotype in non-neuronal cell types, suggesting that it is required for cell-to-cell spread in a variety of cell types [199,201]. In contrast, Us9 null mutants form plaques that are comparable in size to those formed by wild-type PRV [190]. Overall, these results imply that gE/gI and Us9 have distinct functions for mediating anterograde transneuronal spread.

Us9 is a small (106 amino acid) type II tail-anchored membrane protein that is expressed with late kinetics and is enriched within detergent resistant membrane microdomains, known as lipid rafts [202,203]. In the absence of Us9, viral particles are assembled in the cell body, but are unable to sort into axons (Figure 3) [102,108]. Moreover, the Us9 null phenotype is neuron-specific. A long held hypothesis had been that Us9 functions by interacting directly or indirectly with a kinesin motor or cellular adaptor complex that facilitates axonal sorting and transport [124,204]. Despite numerous attempts to identify viral and host proteins that interact with Us9, the molecular mechanism underlying its function remained elusive for many years [108,124,203]. We recently reported that Us9 interacts with KIF1A, a member of the kinesin-3 family of microtubule-dependent molecular motors. Our results imply that KIF1A is also required for axonal sorting and long-distance transport of viral particles towards egress sites in axons. Multiple known properties of KIF1A function are consistent with its role in mediating axonal sorting and transport of viral particles. First, KIF1A is highly expressed in neuronal tissues and is enriched in axons [205,206], thus making it an excellent candidate for facilitating anterograde spread of neurotropic alphaherpesviruses. Second, KIF1A has a well-established functional role in axonal sorting and transport of cargoes, such as synaptic vesicle precursors and dense core vesicles [207]. Several laboratories have reported that loss of KIF1A leads to a reduced number of synaptic vesicle precursors in axons and accumulation of these organelles in cell bodies [208–210]. The Us9 null phenotype is strikingly similar [108,203]. Finally, KIF1A is a relatively fast kinesin motor, with average velocities of 1.2–2.5  $\mu\text{m/s}$ , depending on the model system [211]. The measured average velocity of anterograde-directed PRV capsids in axons is within this range (1.97  $\mu\text{m/s}$ ) [212]. Similarly, the velocity of anterograde transport of VZV particles has been estimated at 1.5  $\mu\text{m/s}$  [213]. However, we cannot exclude the possibility that other kinesin motors are involved in axonal transport of viral particles after axonal sorting. If so, recruitment of additional motor proteins is likely independent of Us9.

**Figure 3.** PRV Us9 and gE/gI mediate anterograde spread of infection in neurons. PRV Us9 is essential for anterograde trans-neuronal spread *in vivo* and *in vitro*. In the absence of Us9, viral particles are assembled in the cell body, but are not sorted into axons. This neuron-specific phenotype suggests that Us9 functions by recruiting a molecular motor protein, either directly or indirectly, that facilitates axonal sorting of viral particles into axons. In contrast, gE/gI null mutants are defective for anterograde trans-neuronal spread *in vivo*, but are able to spread with reduced capacity *in vitro*. gE/gI null mutants display a small plaque phenotype in non-neuronal cell types.



Our results also imply that at least one additional kinesin motor is required for transport of enveloped viral particles in neuron cell bodies and non-neuronal cells. The evidence for this is as follows: (1) Retrograde transneuronal spread is unaltered during infection with PRV strains that are Us9 null [190]. (2) The Us9 null phenotype is neuron specific. Cell types that do not express KIF1A, such as epithelial cells, are susceptible and permissive to PRV infection and are capable of facilitating cell-to-cell spread of infection [124]. Both of these observations necessitate active transport of viral components towards sites of egress at the plasma membrane of cell bodies. (3) Previous studies have implicated other motor proteins in intracellular transport of viral structural components in non-neuronal cells [73,101,167,214]. Unlike KIF1A, which is expressed exclusively in neurons, these motors are expressed in a variety of cell types [143,215]. While the viral and host proteins that mediate these transport events are still unknown, kinesin-1 represents an excellent candidate, both because of its well-characterized function in transporting a broad range of cellular cargoes and its ubiquitous expression in virtually all mammalian cell types [215].

Viral membrane-associated proteins represent the most likely candidates for recruiting motor protein subunits to enveloped virions. In this case, deletion of these viral membrane proteins would produce a phenotype in which virions are assembled, but remain stationary and accumulate within cell bodies of non-neuronal cells (following transfection of mutant viral DNA). However, it has also been proposed that non-membrane associated viral proteins, such as UL36, may also act as receptors for kinesin motors following secondary envelopment [11,167]. This model is dependent upon UL36's putative localization on the cytoplasmic surface of the transport vesicle surrounding intracellular enveloped viral particles, in addition to its well-characterized localization within the tegument layer of virions. Finally, it is also possible that cellular proteins mediate recruitment of kinesin-1 or other motors (besides KIF1A) onto enveloped viral particles.

### *3.5. Reorganization of the Cytoskeleton and Alteration of Organelle Dynamics During Infection*

Alphaherpesvirus infections induce alterations of actin filaments, microtubules and other cellular organelles in a variety of cell types [21,133,134]. For example, the viral kinase, Us3, promotes disassembly of actin stress fibers and outgrowth of cellular protrusions that are proposed to enhance cell-to-cell spread and viral egress [216]. Consistent with this, Roberts and Baines demonstrated that the actin-dependent motor myosin Va has a role in targeting viral membrane glycoproteins to the plasma membrane [214]. Alphaherpesvirus proteins that affect the microtubule cytoskeleton include HSV-1 ICP0, a ring finger E3 ubiquitin ligase that plays a key role in establishing infection. ICP0 is multifunctional and has been shown to bundle and dismantle microtubules in transfected or HSV-1 infected Vero cells [217].

In neurons, altered or aberrant neuronal firing activity leads to reorganization of key cytoskeletal components and changes in organelle dynamics. Two prominent examples are modification of the size and localization of the axon initial segment, as well as alteration of mitochondrial dynamics in response to increased action potential firing [218–222]. These changes have been implicated in a variety of neurological diseases and disorders [223,224]. Our lab previously reported that action potential firing rates dramatically increase beginning at 8–10 hours post PRV infection in cultured rat superior cervical ganglion (SCG) neurons [225]. Consequently, this results in elevated intracellular

$\text{Ca}^{2+}$  and alters mitochondrial dynamics through a mechanism involving the  $\text{Ca}^{2+}$ -sensitive protein, Miro, a cellular protein that regulates mitochondrial motor activity [218,220,226]. Our findings suggest that disruption of mitochondrial motility is important for efficient spread of infection. We hypothesized that this could be due to removal of kinesin-1 from mitochondria for efficient transport of viral particles and, consequently, spread within and between hosts [226]. This hypothesis was based on two observations: (1) kinesin-1 recruitment to mitochondria through Miro is disrupted during infection and (2) PRV overexpressing  $\text{Ca}^{2+}$ -insensitive Miro proteins (PRV Miro1 $\Delta$ EF) is deficient for spread in both PK15 cells and SCG neurons. However, our findings do not exclude the possibility that disruption of mitochondrial motility may be important for other aspects of viral infection and spread (besides viral particle transport). One possibility is that disruption of mitochondrial dynamics through Miro's  $\text{Ca}^{2+}$ -sensitive function may be important for maintaining cell viability by blocking apoptosis. In neurons, elevated intracellular  $\text{Ca}^{2+}$  results in excitotoxic stress that leads to cell death [227]. It is remarkable that neurons cultured *in vitro* are able to survive for several days post infection [225] and do not die of excitotoxic  $\text{Ca}^{2+}$  overload. Alternatively, disruption mitochondrial dynamics during infection may interfere with cellular antiviral signaling cascades that are mediated by mitochondrial membrane proteins. For example, activation of retinoic acid-inducible gene I-like receptors (RLRs) promotes elongation of the mitochondrial network. During infection, this is important for signal transduction through the mitochondrial antiviral signal (MAVS) protein, which triggers the production of type I interferons and proinflammatory cytokines. Castanier *et al.* previously demonstrated that a human cytomegalovirus (HCMV) antiapoptotic protein that promotes mitochondrial fragmentation inhibits signaling downstream from MAVS [228]. This may be a novel HCMV immune modulation strategy.

Thus, disruption of cytoskeletal and organelle dynamics during infection may be necessary for mediating efficient viral spread. Further work is needed to identify additional intracellular changes that occur during infection and determine their potential role in promoting viral replication, growth and spread.

#### 4. Concluding Remarks

Directional movement of alphaherpesvirus infection in the nervous system is essential for replication and spread within and between hosts. Efficient spread of infection in and out of the nervous system is dependent upon long distance transport of viral particles in axons and dendrites. Transport of viral particles, like other cargoes, requires the microtubule-dependent molecular motors. Retrograde transport is dependent on interactions between incoming capsids and tegument proteins with dynein motors. For anterograde spread of infection, plus-end directed kinesin motors facilitate movement of viral particles towards distal sites of egress. The protein composition of viral particles determines which motors are recruited and activated, thus determining the destination of these particles within infected cells. Additional work is needed to understand how viral complexes utilize host proteins for efficient long distance transport within their hosts. In addition, future studies will explore the significance of alterations to cellular organization, dynamics and homeostasis that occur as a consequence of infection. These types of studies are necessary for understanding the nature of alphaherpesvirus spread and pathogenesis in the nervous system.



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## Conflict of Interest

The authors declare no conflict of interest.

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